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Effects of four CeO_2 nanocrystalline catalysts on early-life stages of zebrafish *Danio rerio* and crustacean *Daphnia magna*

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ABSTRACT

Effects of four different nanocrystalline CeO_2-based catalysts on crustaceans *Daphnia magna* and early-life stages of zebrafish *Danio rerio* were studied. Pure CeO_2 and $CuO–CeO_2$ mixed oxides with a nominal 10, 15 and 20 mol.% CuO content were tested. Pure CeO_2 provoked no effects, but $CuO–CeO_2$ mixed oxides induced some sublethal effects on fish and affected daphnids' survival. The most pronounced effects were found on fish body growth, which was reduced at 10 mg/L in case of CuCe20 and 50 mg/L in cases of CuCe10 and CuCe15. Daphnids' survival was affected above 80 mg/L of CuCe20, while CuCe10 and CuCe15 did not affect daphnids. None of the materials was highly toxic to daphnids and fish in comparison to some other environmental pollutants. Differences in effects between the materials could not be explained by their specific physicochemical properties. This work indicates that more attention should be placed at potential toxicity of nanostructured materials, such as nanocrystalline mixed-oxides.

1. Introduction

In recent years, it has been recognized, that nanomaterials are potentially hazardous to environment, however ecotoxicity of nanocrystalline environmental catalysts has not been sufficiently addressed [1]. This work was focused on nanocrystalline pure cerium oxide (CeO_2) and copper–cerium ($CuO–CeO_2$) mixed oxide catalysts. The catalytic activity of $CuO–CeO_2$ is related to the copper concentration in the catalyst and especially to their state of dispersion. The so-called metal–support interaction between copper and ceria is often regarded as the key factor determining the redox properties and as a result catalytic features of material [2]. $CuO–CeO_2$ catalysts have been reported as active catalysts in numerous heterogeneous reactions, e.g. preferential oxidation of CO in excess H_2 (CO PROX) [2], water-gas shift (WGS) reaction [3], steam reforming of methanol [4,5], oxidation of benzene [6], VOC oxidation [7], H_2O_2 decomposition [8], and also catalytic wet-air oxidation (CWO) of phenol [9].

Currently, the production of mixed oxide $CuO–CeO_2$ catalysts is still at the laboratory scale, but due to their favorable catalytic properties, they are expected to be used in industrial applications. On the other hand, pure CeO_2 is already being used in a variety of applications, among them emission control systems in automobile engines as a diesel fuel-borne catalyst to reduce particulate matter emissions [10]. These materials can enter the environment also during accidental spills when uploading/unloading the reactors and during transport. Therefore, the occurrence of CeO_2 and their mixed-oxides in environment is probable, and their potential effects on organisms should be known prior to their wide application.

There are many indications, that metal oxide-based nanomaterials are potentially hazardous to aquatic organisms [11]. Among them, ZnO and CuO nanoparticles are of particular concern. CuO nanoparticles were found toxic to protozoa *Tetrahymena thermophila* (24h EC50 = 100 mg/L) [12], crustaceans *Daphnia magna* (48 h LC50 = 3.2 mg/L); bacteria *Vibrio fischeri* (30 min EC50 = 79 mg/L), *Thamnocephalus platyurus* (24 h LC50 = 2.1 mg/L) [13], algae *Pseudokirchneriella subcapitata* (72 h EC50 = 0.7 mg/L) [14], and zebrafish larvae (96 h LC50 = 0.242 mg/L) [12–15]. Their toxicity was mainly attributed to solubilized Cu and Zn ions, which dissolved from nanoparticles [13]. Therefore, the aspect of potential dissolution of Cu^{2+} from $CuO–CeO_2$ will be addressed in the present paper.

Crustaceans *D. magna* and fish *Danio rerio* are among those aquatic organisms commonly applied to assess the potential hazard of chemicals. Tests on fish and crustaceans are also among a base set ecotoxicity data requested by the EU chemical regulation REACH [16]. As opposed to conventional adult fish toxicity testing, alternative methods, such as those using fish early-life stages (embryos and larvae before the onset of exogenous feeding) and cell lines [17] are preferred. Numerous toxicity studies have shown that zebrafish *D. rerio* embryo test is a possible surrogate for the acute adult fish

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toxicity test [17]. Zebrafish have also become a major model in neurobiology, toxicology, molecular and developmental biology [18] as well as pre-clinical screening of nanopharmaceuticals [19].

In the present study, the effects of four nanocrystalline CeO_2-based catalysts with different physico-chemical properties on crustaceans *D. magna* and early-life stages of zebrafish *D. rerio* were studied. The aim was to assess potential toxicity of tested materials for selected aquatic species and to evaluate the difference in toxicity of pure cerium oxide (CeO_2) in comparison to CuO–CeO_2 mixed oxide nanomaterials.

2. Materials and methods

2.1. Synthesis of nanocrystalline CeO_2 and mixed oxide CuO–CeO_2 catalysts

Nanocrystalline pure CeO_2 and mixed oxide CuO–CeO_2 catalysts with a nominal 10, 15 and 20 mol.% CuO content (named CuCe10, CuCe15, and CuCe20) were synthesized by hard template method using KIT-6 silica. Detailed information on the synthesis can be found in Djinović et al. [20], here only details on possible impurities are provided. $Cu(NO_3)_2{\cdot}3H_2O$ (99.5% purity, Merck; reported impurities in w/w: 0.0005% Cl, 0.005% SO_4, 0.005% Ca, 0.002% Fe, 0.01% K, 0.01% Na, 0.001% Ni, 0.001% Pb, 0.001% Zn) and $Ce(NO_3)_3{\cdot}6H_2O$ (99% purity, Aldrich) were used as precursors for the synthesis. Possible impurities resulting from the synthesis were nitrate species originating from $Cu(NO_3)_2{\cdot}3H_2O$, ethanol and NaOH (Merck). The NO_3^- and ethanol were completely decomposed by heating in an oven [20]. Traces of NaOH were removed by continuously washing samples with distilled water and centrifugation until pH value reached 7.

2.2. Physico-chemical characterization of nanomaterials

Chemical composition of mixed oxides (mol.% of CuO, w/w, % of Cu^{2+}) as well as residual amounts of silica (Si) and sodium (Na) were determined by means of ICP-MS (inductively coupled plasma-mass spectrometry) (Agilent Technologies, model 4500 plus, USA). Powder X-ray diffraction (XRD) diffractograms of CuO–CeO_2 catalyst samples were recorded on a PANalytical X'pert PRO diffractometer using Cu Kα radiation ($\lambda = 0.15406$ nm). Samples were scanned in the 2θ ranges between 0.5° and 5° and 10° and 85° with 0.017° and 0.034° increments, respectively, and recording time of 1 s at each increment. From these data average (1 1 1) CeO_2 crystallite size was calculated with the Scherrer equation. BET (Brunauer–Emmett–Teller) specific surface area measurement and porosity determination (pore volume) were performed using a Micromeritics ASAP 2020 MP/C apparatus. The sizes of nanomaterials' particles were inspected by a field emission scanning electron microscope (FE-SEM, Supra 35 VP, Carl Zeiss, Germany), at an accelerating voltage of 1 kV. The materials were inspected before and after being dispersed in toxicity test media.

The sizes of nanomaterials' particles in aqueous solutions of 0.75 mM $NaHCO_3$ and ISO medium for daphnids were inspected using a dynamic light scattering technique (Microtrac S3500, UK). The concentration of all nanomaterials during these measurements was in the range of 10–500 mg/L. The size was inspected immediately after preparation. In case of ISO daphnid's medium, the size of particles was also measured 2 h and 2 days after preparation to investigate if the size of particles changes during the test. The latter was tested in solutions without the presence of animals, because the volume of the test medium was not sufficient for DLS measurements. Also, the remnants of the moults may affect the measurements.

2.3. Preparation of nanomaterials' dispersions

Nanomaterials were dispersed in 0.75 mM $NaHCO_3$ solution prepared in milliQ water (pH = 7.3). This solution is salt-depleted in comparison to the standard ISO medium [21] and was previously used for testing of other nanoparticles, e.g. TiO_2 nanoparticles, because their aggregation in 0.75 mM $NaHCO_3$ is much lower than in ISO medium (unpublished data). The survival and fitness of control organisms in this solution was the same as in the ISO medium, therefore this medium was used in our subsequent experiments. The solutions were always done in the same manner and were freshly prepared prior to the set up of experiment. The concentrations tested were 1, 10, 50, 100, 250 and 500 mg/L. Each concentration was prepared separately, with the exception of 1 and 10 mg/L, which were prepared by diluting the 100 mg/L solution. The suspensions were first stirred for 1 h on a magnetic stirrer at room temperature, afterwards they were sonicated for 1 h using an ultrasonic bath. Prior to application onto a micro well plate, the suspensions were mixed again on a magnetic stirrer for 1 min. We exposed the organisms immediately after the temperature of the suspension was appropriate for testing (within 10 min). At the end, the micro well plate was shaken for 10 s using a vortex (IKA, Genius 3).

The dissolution of Cu^{2+} from CuO–CeO_2 solids when dispersed in test media was investigated. The suspensions of nanomaterials (50, 100 and 500 mg/L) were prepared exactly the same way as done for fish tests (dispersed in 0.75 mM $NaHCO_3$, see the following paragraph) and daphnids medium (200 mg/L). The suspensions were centrifuged at $80{,}000 \times g$ for 30 min (Beckman Ultracentrifuge). The supernatants were inspected using a SEM (FE-SEM, Supra 35 VP, Carl Zeiss, Germany) and no particles were found in the solution. The pH of all suspensions was between 7.4 and 7.9. Cupric ions in supernatant were determined using an ICP-MS (inductively coupled plasma-mass spectrometry) (Agilent Technologies, model 4500 plus, USA) with detection limit of 0.001 mg/L.

2.4. Toxicity to early life stages of zebrafish D. rerio

Zebrafish breeding to obtain eggs and toxicity tests were performed according to Tišler et al. [22] with slight modifications. Adult zebrafish were bred in a temperature-controlled room in aquarium (60 cm × 30 cm × 30 cm) containing 45 L of tap water with constant temperature (26 °C) and photoperiod (12 h light:12 h dark). A day before breeding a plastic spawning box covered with stainless steel mesh was placed in the breeding tank. On the following day, 1 h after the light cycle started, the spawning plastic box was removed from the tank and eggs were collected.

Fertilized eggs in the four to eight cell stages were placed in 24-well plates; each well contained 1 mL of test media and 1 egg. In each test, 10 eggs per control containing only 0.75 mM $NaHCO_3$ solution and 10 eggs per each concentration of nanomaterial's suspension were exposed. The plate was covered with a transparent plastic self adhesive foil to prevent the evaporation of medium. The test suspensions were renewed at 48 h intervals. Larvae were not fed during the test according to OECD 212 [23]. For each of the four nanomaterials, the test was repeated three times. After 24 and 48 h of exposure malformations of embryos were evaluated [22] and after the embryos have hatched, every day onwards (up to 7 days post fertilization) the larvae were being observed for the mortality, malformations and body length using a stereomicroscope WILD M7 (Heerbrugg, Germany).

Along with a negative control containing only 0.75 mM $NaHCO_3$ solution, also a positive control with the reference chemical 3,4-dichloroaniline was always prepared to check for the sensitivity of embryos. The concentrations tested were 2, 2.5, 3 and 3.7 mg/L. The sensitivity of embryos between the experiments did not differ,

Table 1
Chemical composition, morphological and structural properties of nanocrystalline CeO_2, CuCe10, CuCe15 and CuCe20 materials [20].

Measurement	Method	CuCe10	CuCe15	CuCe20	CeO_2
CuO, mol.%	ICP-MS	9.2	14	18	/
Cu^{2+}, % (w/w)	ICP-MS	3.5	5.3	7.2	/
Si, % (w/w)	ICP-MS	1.3	1.8	1	/
Na, % (w/w)	ICP-MS	0.15	<0.01	0.07	/
BET surface area, m^2/g	BET	147	166	161	134
Pore volume, cm^3/g	BET	0.29	0.31	0.33	0.30
Average (1 1 1) CeO_2 crystallite size, nm	XRD	7.8	6.5	6.6	8.2
Average CuO particle size, nm	Calculated from selective pulse N_2O decomposition	1.3	1.9	1.7	/

/, not determined; BET, Brunauer–Emmett–Teller surface area analysis.

since the 24 and 48 h LC50 (based on at least one of the lethal malformations) were always within the narrow ranges: 2.2–3.0 mg/L and 2.3–2.6 mg/L, respectively.

The cumulative hatching rate, hatching success of larvae and total occurrence of malformations were calculated as follows. The hatching rate (%) is the number of all hatched embryos divided by the total number of eggs exposed. The hatching success of larvae (%) is the number of larvae alive divided by the total number of larvae that have hatched. The total occurrence of malformations (%) is the percentage of larvae with at least one malformation (either spine deformation or pericardial oedema). The body length was measured as a distance from the most anterior part of the head to the tip of the tail, following the path of a developing spinal cord. Larvae with spine deformities were not inspected for the length. A Nikon DS-Fi1 digital camera and a NIS-Elements Documentation 2.2 imaging software were used to measure the body length.

2.5. Acute toxicity to crustaceans D. magna

Water fleas *D. magna* Straus 1820 were obtained from the Institut für Wasser, Boden und Lufthygiene, des Umweltbundesamtes (Berlin). They were cultured in 2.5 L of modified M4 media at 21 ± 1 °C and 16:8 h light/dark regime (1800 lux) with a diet of the algae *Desmodesmus subspicatus* Chodat 1926.

Our laboratory is accredited for standard acute testing with *D. magna*. Quality of the test results is regularly assured by the internal quality control (control charts) and participation in proficiency testing schemes (AQUACHECK, UK), where good performance has been demonstrated. The appropriate sensitivity of *D. magna* is regularly checked using a reference chemical potassium dichromate according to the ISO 6341: 1996 [24].

The tests were done according to ISO 6341:1996 [24]. In brief, neonates less than 24 h old, derived from the second to fifth brood, were exposed to nanomaterials' suspensions. A preliminary test and two definite trials were done for each nanomaterial. Twenty daphnids per concentration were exposed in each experiment. After a 24 h and 48 h exposure period the immobile daphnids were counted. Immobile daphnids were considered as those which would not swim within 15 s after gentle agitation. The moult rate of daphnids was evaluated by counting the moults after 24 h and 48 h. The moult rate was calculated as the number of observed moults divided with expected number of moults in case all exposed animals would moult (20 moults at each concentration of each experiment).

The suspensions of nanomaterials were prepared in daphnids' ISO medium [24]. The concentrations tested for all four nanomaterials were 100, 150 and 200 mg/L, while in the case of CuCe20 sample also lower concentrations 10, 50 and 80 mg/L were tested. Each concentration was prepared separately and freshly prior to experiments. The suspensions were first stirred for 1 h on a magnetic stirrer at room temperature, afterwards they were sonicated for 1 h using an ultrasonic bath.

2.6. Statistical analysis

One-way Analysis Of Variance (ANOVA) with a Mann–Whitney post hoc test were used to test the differences between the control and different concentrations of nanomaterials ($p < 0.05$). Lowest-observed effect concentration (LOEC) was determined as the lowest concentration producing statistically significant response. All tests were done using Statgraphics software (Statgraphics Plus for Windows 4.0, Statistical Graphics Corporation).

3. Results

3.1. Physico-chemical properties of nanomaterials

Since CuO particles are of nanometer size and highly dispersed, their addition to CeO_2 actually increased the surface area and total pore volume, therefore pure CeO_2 exhibited lower BET surface area than mixed oxides. Among the former, CuCe10 catalyst had lower surface area than CuCe15 and CuCe20 solids, which exhibited similar areas (Table 1).

Average CeO_2 crystallite size, determined by wide angle XRD analysis, is very similar to the pore diameter of the parent template, thus confirming their entrapment in the template during the mineralization process which limited their growth. It also confirmed minute size and/or amorphous structure of CuO entities, which were invisible during this analysis. Low angle XRD characterization revealed ordered domains in the mesostructure of the prepared CuO–CeO_2 powders, but also indication of some structure collapse during template removal (Table 1).

SEM micrographs revealed, that particles of all four materials are very irregularly shaped and polydisperse in diameters up to several tens of micrometers (available in Supporting information). No differences between the four nanomaterials could be found. Also, no change in the size of particles after being dispersed in toxicity test media was observed by SEM.

The median sizes of nanomaterials (D50) obtained by dynamic light scattering analysis were in the range of 9.15–9.95 μm when dispersed in 0.75 mM $NaHCO_3$, and in the range 8.28–10.73 when dispersed in ISO medium for daphnids (Table 2). It seems that particles of CeO_2 are slightly larger than the three mixed oxides, in particular in ISO medium. We consider the differences between the three mixed oxides being in range of experimental error. D50 values were found to be independent of nanomaterial concentration (10–500 mg/L). For the purposes of comparison of DT50 values for all four materials, data at 100 mg/L are shown (Table 2). The size of particles was not significantly changed during 2 days in ISO medium.

In case of 50 mg/L, 100 mg/L and 200 mg/L of all three nanomaterials no dissolved Cu^{2+} was found (<0.001 mg/L of Cu^{2+}). At 500 mg/L, the concentrations of Cu^{2+} were 0.041 ± 0.013 mg/L, 0.045 ± 0.014 mg/L and 0.058 ± 0.017 mg/L for CuCe10, CuCe15 and CuCe20, respectively.

Table 2
The sizes of nanomaterials' particles in aqueous solutions: 0.75 mM $NaHCO_3$ and ISO medium for daphnids (100 mg/L) [24]. For the latter, also the effect of time on the size of nanomaterials was investigated 2 h and 2 days after preparation.

	Distribution values	0.75 mM $NaHCO_3$ (pH = 7.27)	ISO medium[a] (pH = 7.52)	ISO medium[a] (pH = 7.52)	ISO medium[a] (pH = 7.52)
Time of measurement		Within 1 h of preparation	Within 1 h of preparation	2 h after preparation	2 days after preparation
CeO_2	D10 (μm)	1.704	2.099	1.956	2.045
	D50 (μm)	9.95	10.73	10.54	10.41
	D90 (μm)	21.31	24.16	23.22	22.42
CuCe10	D10 (μm)	2.350	1.612	1.567	1.612
	D50 (μm)	9.15	8.28	8.02	7.97
	D90 (μm)	18.91	20.66	20.53	20.01
CuCe15	D10 (μm)	2.192	1.778	1.699	1.752
	D50 (μm)	9.57	9.17	8.99	8.92
	D90 (μm)	20.96	20.96	21.68	21.78
CuCe20	D10 (μm)	1.965	1.832	1.854	1.979
	D50 (μm)	9.53	8.83	9.04	9.03
	D90 (μm)	33.80	20.35	24.57	20.56

D10 – 10% of values lies below this size; D50 – median; 50% of values lies below this size; D90 – 90% of values lies below this size.
[a] Reference to [24].

3.2. Effects on early life stages of zebrafish D. rerio

None of the four tested nanomaterials caused the mortality of embryos, while the effects on larvae after hatching were found. Pure CeO_2 did not affect any of the parameters, but mixed oxides provoked some sublethal effects (Table 3, Figs. 1–3). More pronounced effects were found after 7 dpf in comparison to 4 dpf. After 4 days only CuCe10 affected hatching success at very high concentration (500 mg/L). However, at 7 dpf both CuCe10 and CuCe20 reduced hatching success (LOEC = 100 mg/L), while no effect of CuCe15 on hatching success was found (Fig. 1, Table 3). All CuCe mixed oxides caused malformations at 7 dpf (LOEC = 100 mg/L for CuCe10 and CuCe15), but in case of CuCe20 the response was not dose-dependent and LOEC could not be determined (Fig. 2, Table 3). Body length was the most sensitive parameter, because already at 4 dpf fish body growth significantly decreased in case of all three materials (Fig. 3, Table 3). According to this parameter, CuCe20 was the most toxic with a LOEC of 10 mg/L.

The frequency of malformations increased with time of observation; significantly more pronounced malformations were found after 7 dpf in comparison to 4 dpf (Fig. 2). After 7 dpf the occurrence of malformations was very high, for example up to 60% of specimen was deformed at 500 mg/L of CuCe10, 27% at 500 mg/L of CuCe15 and 39% at 250 mg/L of CuCe20. The most common types of malformations were spine malformations and pericardial oedema (Fig. 4). Among spine malformations most commonly we observed altered axial curvature and malformed tail. No other malformations, such as opaque yolk, opaque head region, and submandibular edema were observed.

The experiment with each of the nanomaterials was repeated three times and variation of results was assessed by estimating standard deviation. Cumulative hatching rate was found as the least reliable parameter, because very high standard deviations (on average 39%) were found. Significantly lower standard deviations were found for hatching success (on average 6%) and occurrence of malformations (on average 7%). Therefore, data on hatching rate were excluded from further data analysis.

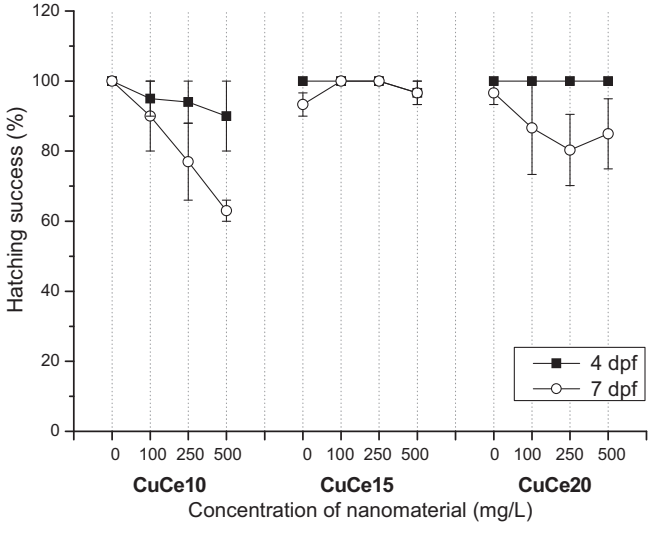

Fig. 1. Hatching success of zebrafish (survival of larvae) exposed to 100, 250 and 500 mg/L of CuCe10, CuCe15 and CuCe20 at 4 and 7 days post fertilization (dpf) (mean ± SE). No effect on hatching success was observed at 1, 10 and 50 mg/L of nanomaterials.

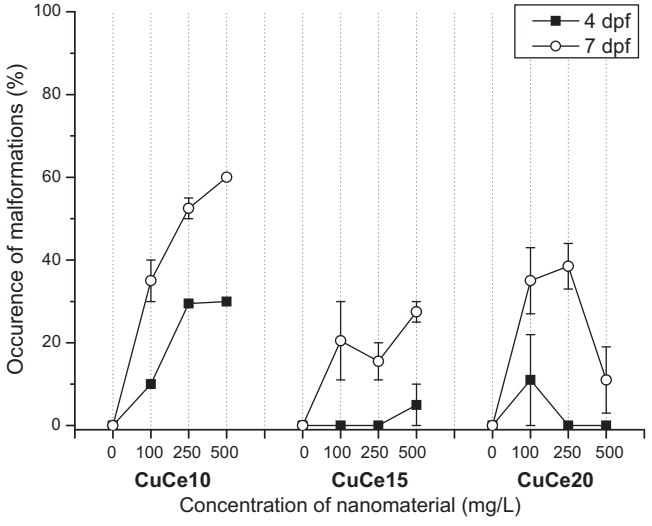

Fig. 2. Occurrence of zebrafish malformations exposed to 100, 250 and 500 mg/L of CuCe10, CuCe15 and CuCe20 at 4 and 7 days post fertilization (dpf) (mean ± SE). No malformations were observed at 1, 10 and 50 mg/L of nanomaterials.

Table 3
The summary of 4 days and 7 days LOEC values (mg/L) for parameters in fish larvae.

Material/LOEC values (mg/L)	Hatching success		Malformations		Body length	
	4 days	7 days	4 days	7 days	4 days	7 days
CeO_2	–	–	–	–	–	–
CuCe10	500	100	100	100	50	≤50
CuCe15	–	–	–	100	50	≤50
CuCe20	–	100	n.d.	n.d.	10	≤10

n.d. could not be determined due to lack of dose-dependent response; – no effect up to 500 mg/L.

3.3. Toxicity to crustaceans D. magna

No effects of CeO_2, CuCe10 and CuCe15 on daphnids' immobility or moult rate were observed. However, CuCe20 sample caused immobility of daphnids up to 200 mg/L after 48 h of exposure. The percentages of immobile daphnids with corresponding standard error of mean were: 5 ± 5, 5 ± 5, 10 ± 0, 10 ± 0, and 10 ± 0 after 24 h and 10 ± 0, 10 ± 0, 40 ± 0, 35 ± 5, and 45 ± 5% after 48 h at 10, 50, 80, 100, and 200 mg/L of CuCe20, respectively.

The moult rate of daphnids was evaluated. After 24 h moult rate in control was 0%, in other treatments no difference in comparison to control or slightly higher rate (10%) was found. After 48 h, 90–100% of daphnids moulted in case of CeO_2, CuCe10 and CuCe15, which was similarly to control moult rate. After 48 h moult rate in CuCe20 exposed daphnids was decreased in experiment 1, but this was not confirmed in experiment 2. We therefore conclude, that moult rate is not a reliable parameter to assess the effects of nanomaterials on daphnids. The decrease of moult was not related to immobility, for example after 48 h moult was more affected at

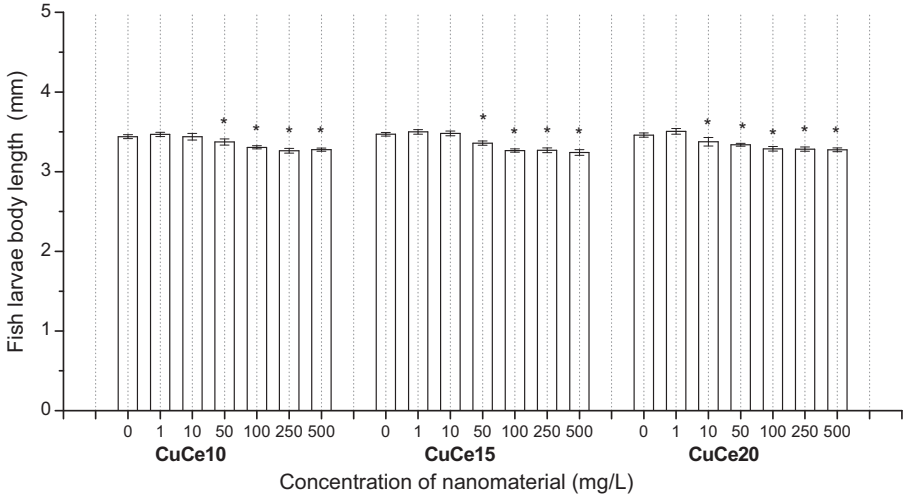

Fig. 3. The body length of fish exposed to CuCe10, CuCe15 and CuCe20 in comparison to control (100%) at 4 dpf (mean ± SE). Statistically significant differences in comparison to control are shown (*$p < 0.01$).

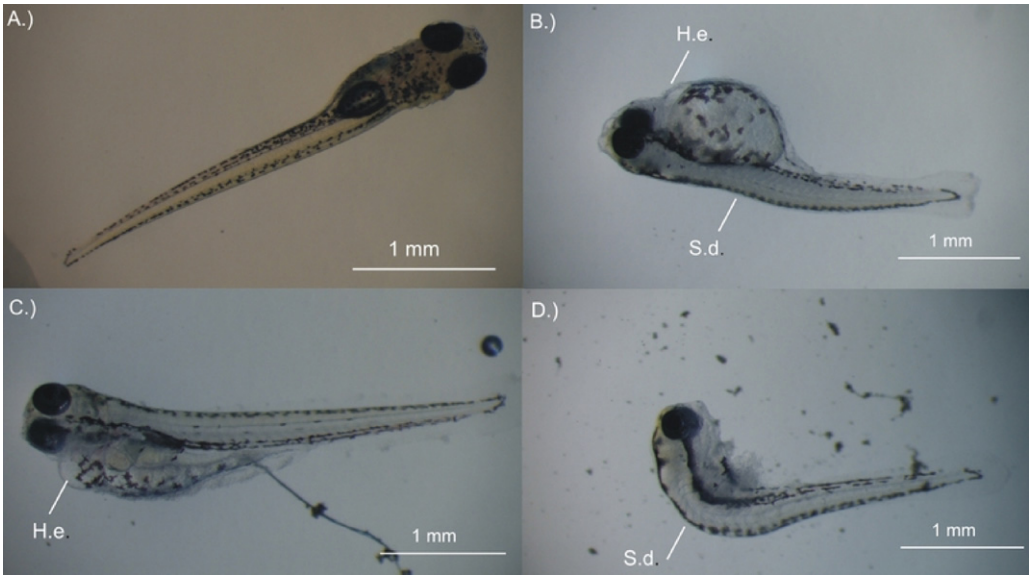

Fig. 4. Different types of larval malformations after 7 days post fertilization: (a) control, (b–d) exposed to CuO–CeO_2 mixed oxides (h.e. – heart edema; s.d. – spine deformation).

Table 4
Immobility of daphnids and corresponding moult rate in two separate experiments with daphnids exposed to CuCe20 for 24 h and 48 h.

CuCe20 (mg/L)	Experiment 1				Experiment 2			
	Immobility (%)		Moult rate[a] (%)		Immobility (%)		Moult rate[a] (%)	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Control	0	0	0	90	0	0	0	90
10	10	10	0	90	0	10	0	90
50	10	10	10	20	0	10	0	90
80	10	40	10	30	10	40	10	90
100	10	30	10	40	10	40	10	100
200	10	50	10	10	10	40	10	100

[a] Number of observed moults after 24 h and 48 h divided with expected number of moults in case all exposed animals would moult (20 moults at each concentration of each experiment).

50 mg/L were only 10% immobility was observed, but on the contrary more daphnids moulted at 100 mg/L where 30% immobility was found (Table 4).

4. Discussion

We studied the toxicity of four different nanocrystalline CeO_2-based catalysts to two aquatic species, daphnids *D. magna* and fish *D. rerio*. In general, we can conclude that none of the materials is highly toxic to tested aquatic organisms in comparison to some other environmental pollutants [17,25,26]. For example, pesticide diazinon is considered highly toxic to daphnids with a 48 h EC50 = 0.0061 mg/L and Cr^{6+} induces such effects at 0.36 mg/L [25]. Only CuCe20 caused immobility of daphnids at considerably higher concentration (48 h LOEC = 80 mg/L). Also, in case of fish, no lethal effects on embryos were observed up to 500 mg/L which indicates low toxicity of these materials in comparison to some pesticides and metals [17].

Among the materials tested, sublethal effects of CuO–CeO_2 mixed oxides on fish were found. The difference in effects between the three mixed oxides was difficult to establish, because the sensitivity of parameters was not the same for all three mixed oxides. For example, based on the hatching success, CuCe15 was the least toxic, based on the occurrence of malformations, CuCe20 was the least toxic, but this material most severely affected body length. It is difficult to predict exactly which of the materials' physico-chemical properties governed the observed effects, since these are the result of an integrated action of more properties under specific conditions. Furthermore, properties of nanomaterials, which are usually inspected in powder form (e.g. BET surface area), are different in aqueous solution where aggregates or agglomerates are formed. Authors usually relate observed toxic effects of nanomaterials to their size and surface area [1,27]. In this study, dynamic light scattering measurements revealed, that CeO_2 particles are larger than their mixed-oxides, in particular in ISO medium. Because there is an inverse relationship between the size of particles and surface area, it is assumed, that CeO_2 particles have smaller surface area than Cu–Ce mixed oxides [1]. Since surface area has previously been shown to influence the toxicity of nanoparticles; higher surface area provoked higher toxicity [27], we could explain the observation why CeO_2 was less toxic in comparison to mixed oxides. However, this could not explain the differences in toxic effects of the three mixed-oxides, which proved to be of similar sizes as inspected by DLS.

One possible factor affecting the toxicity of nanomaterials is the dissolution of metals from metal oxides [28]. The dissolution of Cu^{2+} from CuCe nanomaterials dissolved in 0.75 mM $NaHCO_3$ was observed only at the highest concentrations tested (500 mg/L) and is considered low (0.16–0.23% is in dissolved form, 0.041–0.058 mg/L of Cu^{2+} was found). However, cupric ions are highly toxic to daphnids (48 h EC50 = 0.032 ± 0.0029 mg/L) [29] and zebrafish [30] (hatching, hearth rate, body length were decreased after 72 h exposure to 0.05 mg/L of Cu^{2+}). Therefore, at 500 mg/L of nanomaterials, Cu^{2+} could be involved in observed effects on fish and daphnids, but this could not be the sole reason, because the effects on these organisms were also observed at lower concentrations of materials, where no dissolved Cu^{2+} was found.

It has been previously suggested, that the cytotoxicity of CeO_2 nanoparticles to bacteria *E. coli* depends largely on a direct contact of CeO_2 with bacteria *E. coli*, which causes a reduction of Ce^{4+} to Ce^{3+}, the latter being the main cause of toxicity [31]. Van Hoecke et al. [27] on the contrary found negligible dissolution and reduction of Ce^{4+} in OECD algae medium. The materials tested in this work reduce from Ce^{4+} to Ce^{3+} in gas free atmosphere above 100 °C and 400 °C in case of CuO–CeO_2 mixed oxides and pure CeO_2, respectively [32]. Therefore, it is not expected, that these materials reduce from Ce^{4+} to Ce^{3+} under toxicity test conditions in the present study.

Impurities of nanomaterials were ruled out as a possible cause for effects. Ethanol and NO_3^- were completely removed during the synthesis of solids [20]. The highest concentrations of Si and Na in fish tests were 9 mg/L and 0.75 mg/L, respectively, and 3.6 mg/L and 0.3 mg/L, respectively in daphnids' test. Silica is chemically and biologically inert and is not expected to be toxic and also SiO_2 nanoparticles were found non-toxic in this concentration range [33]. Traces of Na could not have been toxic considering the fact that this concentration is about 23 times lower than the one present in control test media for both organisms (0.75 mM $NaHCO_3$).

Significant variability in fish hatching rate was found when the experiment was repeated with the same nanomaterial. A number of positive and negative controls confirmed, that this is not a consequence of methodological error. Also, the rest of parameters exhibited significantly lower variations. Hatching seem to be largely dependent on the extent of entrapment of embryos in the sediment of the nanomaterials' suspension. Namely, although a number of steps were taken to prepare stable suspensions (i.e. medium with low ionic solution, sonication, stirring), the materials settled down soon after application. We could observe that nanomaterials bind to the surface of embryos very stochastically, depending on where the embryo would settle (being in the sediment of the materials or not). This implies that hatching rate is not the most reliable parameter when evaluating the toxicity of nanomaterials. In addition, other parameters in fish, e.g. hatching success, malformations and body length, should be analyzed. Also, the time of exposure should be prolonged. As revealed in this work, malformations and mortality of larvae were evidently higher after 7 dpf in comparison to 4 dpf. Prolonged time of exposure has previously also been proposed by others [34,35].

Among end-points evaluated in zebrafish, larval body length proved to be the most sensitive and repeatable biomarker when exposed to nanocrystalline CeO_2 materials. This effect has previously been found in case of nanomaterials [36]. The incidences

of decrease after 4 dpf at the highest concentrations of CuCe10, CuCe15 and CuCe20 solids were 4.7, 6.7 and 5.3%. For comparison of severity of this growth retardation, the control larvae increase their length from 3 dpf to 4 dpf by 4.8%, from 4 dpf to 7 dpf another 5.2% and altogether 10% from day 3 to day 7. It seems that already small changes of growth (less than 5%) are already physiologically relevant. Very small changes in body length were also previously found to be statistically significant in comparison to control. For example, an estimated 3% decrease in body length was found significant ($n = 20$; $p < 0.05$) when zebrafish were exposed to Zn^{2+} [36] and an estimated 5% decrease in body length was found significant ($n = 18$; $p < 0.05$) when exposed to Cu^{2+} [30].

The zebrafish malformations induced by nanocrystalline CuCe materials were spine malformations and pericardial edema. Such malformations seem to be a common response of zebrafish to nanoparticles [30,34–40] and other chemicals besides nanomaterials [41,42]. Pericardial edema is an indicator of a defective cardiovascular system in zebrafish [40]. In a specific study Incardona et al. [43] found that pericardial edema is caused by inhibition of an essential component of the sarcomere in cardiomyocytes and the edema was proceeded by spine deformities. This might be the reason, why these two malformations often occur in combination.

To our knowledge, there is currently no other published toxicity data for nanocrystalline CeO_2-mixed oxide catalysts available. Data on CeO_2 nanoparticles have recently emerged; no toxicity to daphnids [27,33,44] and zebrafish [27] were found, but García et al. [45] found significant effect of CeO_2 nanoparticles (6.5 nm in diameter) on daphnids. No effects of bulk sized CeO_2 (<5 μm) on daphnids were recorded [44]. In contrast to CeO_2 nanoparticles, nanocrystalline Cu–Ce mixed oxide catalysts tested in this work provoked effects on zebrafish and daphnids at 10 mg/L and 80 mg/L, respectively. This indicates that more attention should be placed at potential toxicity of nanostructured mixed oxide materials.

Other commonly applied metal oxide nanocatalysts, such as TiO_2 and Al_2O_3, did not affect zebrafish at early developmental stages [46]. Similar results were observed for CeO_2 in this study, but Cu–Ce mixed oxides were more toxic. As reviewed by Menard et al. [47], extremely variable 48-h EC50 and LC50 values were reported for TiO_2 nanoparticles in *D. magna*, the values ranged from 5.5 mg/L up to 20,000 mg/L. Therefore, the comparison to data obtained in this study is not possible. To our knowledge, no literature data currently exists on the potential toxicity of other metal oxide nanocatalysts (V_2O_5, ZrO_2 and MgO) to daphnids and zebrafish.

In conclusion, nanocrystalline pure CeO_2 and mixed oxide CuO–CeO_2 catalysts are not highly toxic to daphnids *D. magna* and fish *D. rerio* in comparison to some other environmental pollutants, but still some sublethal effects of CuO–CeO_2 mixed oxides on fish were found and daphnids immobility was affected. Currently, very little data on occurrence of these materials in aquatic environment exist, therefore we tested a range of nanomaterials' concentrations (1–500 mg/L). Environmental relevance of such high concentrations remains unclear; however it is possible that during accidental spills high concentrations enter the environment. At the moment, it seems that no severe hazard of these nanomaterials for the environment exists.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2012.03.080.

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